Culture of Human Endothelial Cells Derived from Umbilical Veins

IDENTIFICATION BY MORPHOLOGIC AND IMMUNOLOGIC CRITERIA

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ABSTRACT Endothelial cells were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein. The cells were grown in tissue culture as a homogeneous population for periods up to 5 mo and some lines were subcultured for 10 serial passages. During the logarithmic phase of cell growth, cell-doubling time was 92 h. Light, phase contrast, and scanning electron microscopy demonstrated that cultured human endothelial cells grew as monolayers of closely opposed, polygonal large cells whereas both cultured human fibroblasts and human smooth muscle cells grew as overlapping layers of parallel arrays of slender, spindle-shaped cells. By transmission electron microscopy, cultured endothelial cells were seen to contain cytoplasmic inclusions (Weibel-Palade bodies) characteristic of *in situ* endothelial cells. These inclusions were also found in endothelial cells lining umbilical veins but were not seen in smooth muscle cells or fibroblasts in culture or *in situ*. Cultured endothelial cells contained abundant quantities of smooth muscle actomyosin. Cultured endothelial cells also contained ABH antigens appropriate to the tissue donor's blood type; these antigens were not detectable on cultured smooth muscle cells or fibroblasts. These studies demonstrate that it is possible to culture morphologically and immunologically identifiable human endothelial cells for periods up to 5 mo.

INTRODUCTION

The endothelial cell plays an important role in physiologic hemostasis (1–9), blood vessel permeability (10–12), and the response of the blood vessel to other physiologic and pathologic stimuli (13–19). Abnormalities of endothelial cell structure and function may contribute significantly to diseases of blood vessel walls such as thrombosis, atherosclerosis, and vasculitis (8, 17–19). The roles of endothelial cells in these important biologic events might be better defined if it were possible to isolate and culture them for in vitro study.

Culture of these cells has been attempted by a number of investigators (20–24) over the last few years, but two recurring problems have hampered this work: (*a*) inability to maintain endothelial cells in pure culture for reasonable periods of time; (*b*) inability to identify the cultured cells as endothelium.

Maruyama (20) cultured what were presumed to be endothelial cells from umbilical veins, but the characterization of these cells in culture was incomplete. In this paper we report on the long-term in vitro culture of cells derived from human umbilical veins. These cells have been identified as endothelial cells by morphologic and immunologic criteria.

METHODS

Preparation of cells and cell culture. Endothelial cells were obtained from human umbilical cord veins by an adaptation of the method of Maruyama (20). A sterile technique was utilized in all manipulations of the cord. The cord was severed from the placenta soon after birth, placed in a sterile container filled with cord buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M phosphate buffer, pH 7.4, 0.011 M glucose), and held at 4°C until processing. Storage time averaged about 1 h, and cords were discarded if held more than 3 h. The cord was inspected, and all areas with clamp marks were cut off. The umbilical vein was cannulated with a blunt 14 gauge needle, 2 cm long, and the needle was secured by clamping the cord over the needle with an umbilical cord tie (Nylon ties SST-2, Extracorporeal Medical Specialties, Inc., King of Prussia, Pa.). The vein was perfused with 100 ml of cord buffer to

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wash out the blood and allowed to drain. The other end of the umbilical vein was then cannulated with a blunt, hubless, 12 gauge needle shaft over which was slipped a 4 cm length of $\frac{1}{8}$ in OD polyethylene tubing. 10 ml of 0.2% collagenase (type CLS, Worthington Biochemical Corp., Freehold, N. J.) in cord buffer was then infused into the umbilical vein, and the polyethylene tubing was clamped shut with a hemostat. The umbilical cord, suspended by its ends, was placed in a water bath containing cord buffer and incubated at 37°C for 15 min. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30 ml of cord buffer. The effluent was collected in a sterile 50 ml conical centrifuge tube (2070, Falcon Plastics, Oxnard, Calif.) containing 10 ml of Medium 199 (TC 199) [1] with 20% fetal calf serum (FCS). The cells were sedimented at 250 *g* for 10 min and washed once with 20 ml of TC 199-20% FCS, and the cell button was resuspended by trituration in 5 ml of fresh culture medium. The yield from this procedure was in the range of $0.5–1.5 \times 10^6$ cells. The cell suspension was divided equally among four to six plastic 35-mm Petri dishes (3001, Falcon Plastics). Sufficient medium was then added to make a final volume of 2 ml/dish. Endothelial cells were cultured in TC 199 containing 20% FCS, penicillin (200 U/ml), streptomycin (200 μg/ml), and L-glutamine (2 mM) (Grand Island Biological Co., Grand Island, N. Y.). The dishes were incubated at 37°C under 5% CO_2. The cells were fed twice a week with a complete change of fresh culture medium. For subculture, cells were harvested with 0.01% EDTA-0.1% collagenase.

Smooth muscle cells were cultured from umbilical cord veins by a minor modification of the above technique. Before collagenase perfusion of the vein, the entire cord was traumatized by repeatedly clamping it with a hemostat. The usual procedure was then followed. The effluent cells when cultured yielded a mixed population of endothelial cells and long spindle-shaped cells. These latter cells overgrew the endothelial cells within 2 wk and formed a pure population of spindle-shaped cells growing in multiple layers. They were identified as smooth muscle cells by ultrastructural criteria (25).

Fibroblasts were derived from normal skin obtained at surgery. The starting material was trimmed to remove the epidermis, and the dermal connective tissue was minced into pieces approximately 1×1 mm. The connective tissue fragments were incubated with 0.2% collagenase in cord buffer for 5 h at 37°C. The incubation mixture was centrifuged at 250 *g* for 10 min. The pelleted material was washed three times and resuspended in culture medium, and the suspension was pipetted into 35-mm Petri dishes. Fibroblasts were cultured in Eagles minimal essential medium (Associated Biomedic Systems, Inc., Buffalo, N. Y.) containing penicillin (100 U/ml), streptomycin (50 μg/ml), 10% FCS, and nonessential amino acids (Grand Island Biological Co.). The dishes were incubated at 37°C under 5% CO_2. The cells grew out from many tissue fragments within 2 wk, and by 4 wk the bottom of the Petri dish was well covered by fibroblasts. This monolayer was harvested with 0.01% EDTA-0.1% collagenase in cord buffer and subcultured to other Petri dishes as needed.

Histologic studies. Sections of umbilical cords were taken before and after enzyme treatment and fixed in 10% buffered formalin, pH 7.4. Sections (4 μm) were cut and stained with periodic acid-Schiff and photographed with a Zeiss microscope (Carl Zeiss, New York).

Endothelial cells were grown on 22×22-mm glass cover slips precleaned with 70% ethanol and placed on the bottom of 35-mm diameter Petri dishes. For phase contrast microscopy the cover slips were mounted cell side down on 1×3-in microscope slides with one drop of culture medium, and the cover slip edges were sealed to the slide with silicon high vacuum grease (Dow Corning Corp., Midland, Mich.). For histologic staining the cover slips were fixed in Bouin's solution and then stained with hematoxylin and eosin. Fibroblasts and smooth muscle cells were cultured on cover slips and were processed in the same manner.

Preparation of cells and tissues for microscopy. Cultured cells to be examined by scanning electron microscopy were grown on cover slips in the bottom of plastic culture dishes. Cover slips were removed, and the attached cells were fixed for 6–12 h in 1% glutaraldehyde in 0.15 M phosphate buffer, pH 7.3, washed in several changes of buffer, and postfixed in 1% osmium tetroxide in 0.15 M phosphate buffer for 1 h. The cover slips were rinsed in several changes of buffer and then distilled water, dehydrated sequentially first in a series of graded alcohols and then in a series of mixtures of amyl acetate and alcohol, and then critical point dried in CO_2 at a temperature of 39–41°C and at a pressure of 1,300 lb/in^2. Small 2×2-mm fragments of the tissue-coated cover slips were then glued onto polished aluminum stubs, coated uniformly *in vacuo* with about 200 Å thickness of gold and viewed immediately with a scanning electron microscope (ETEC Autoscan, model U-1, ETEC Corp., Hayward, Calif.).

Cultured endothelial cells to be examined by transmission electron microscopy were prepared by one of two methods. Some cells were prepared by a modification of the method of Ross (25). Cells were grown on the bottom of plastic culture dishes that had been carbon coated in a vacuum evaporator and sterilized in ethylene oxide. Cultured cells were fixed for 20–30 min at room temperature with slight agitation in a solution containing equal volumes of Karnovsky's fixative (26) and fresh culture medium. Fixed cells were washed with fresh culture medium and postfixed for 30 min at room temperature in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3. They were then prestained with 0.5% uranyl acetate in 0.04 M veronal buffer, pH 7.5, for at least 2 h or in some instances overnight, dehydrated in a series of ethanols, and embedded in Epon 812 using the method outlined by Ross (25). Cells were easily identified on the surface of the Epon block with the aid of a dissecting microscope. Ultrathin sections were cut and picked up on 200 mesh uncoated copper grids and restained with uranyl acetate followed by lead citrate. Grids were then lightly carbon coated and viewed in a Siemens Elmiskop 1 (Siemens Corp., Iselin, N. J.). Other cultured endothelial cells were prepared by detaching them from Petri dishes by incubation for 15 min at 37°C with 0.01% EDTA-0.1% collagenase in cord buffer. They were washed three times with 0.1 M cacodylate buffer, pH 7.3, by centrifugation at 500 *g*, fixed for 20 min at room temperature in a solution containing equal volumes of Karnovsky's fixative and 0.1 M cacodylate buffer, and washed with fresh 0.1 M cacodylate buffer. These cells were then processed as above. Cultured smooth muscle cells and fibroblasts were similarly processed.

Segments of umbilical vein were dissected while immersed in 1.5% glutaraldehyde. Small 1–2-mm pieces of

[1] *Abbreviations used in this paper:* AT, antithrombosthenin; AUAM, Antiuterine actomyosin; F-AT, fluorescein-conjugated antithrombosthenin; F-AUAM, fluorescein-conjugated antiuterine actomyosin; FCS, fetal calf serum; PBS, phosphate-buffered saline; TC 199, Medium 199.

vein wall were fixed for 2 h in 1.5% glutaraldehyde in Millonig's buffer (27), and postfixed for 1 h in 1% osmium tetroxide. They were briefly washed in distilled water and stained in 0.5% uranyl acetate in 0.04 M veronal buffer overnight. Blocks of tissue were dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in the Siemens Elmiskop 1.

Preparation of antisera for immunofluorescence studies. Human platelet actomyosin or thrombosthenin, uterine actomyosin, skeletal muscle actomyosin, and cardiac actomyosin were prepared as described previously (9, 28–30). No obvious contaminants were demonstrable in these antigens by polyacrylamide gel electrophoresis. Rabbit antisera to platelet actomyosin or thrombosthenin (AT), uterine actomyosin (AUAM), skeletal muscle actomyosin, and cardiac actomyosin were prepared, and smooth muscle actomyosin monospecificity was demonstrated as previously described (9, 28–30).

Antisera to rabbit IgG were prepared in guinea pigs and goats. Rabbit IgG used as antigen was separated from normal rabbit serum by ammonium sulfate fractionation and chromatography on DEAE-cellulose. Guinea pig or goat antisera to rabbit IgG was tested for the presence of and specificity of antibodies to IgG by immunoelectrophoresis in agarose gel. By this technique, one precipitin arc was found between guinea pig or goat antisera to IgG and whole rabbit serum. The location of this precipitin arc was typical of IgG.

Anti-H lectin was extracted from *Ulex europaeus* seeds in phosphate-buffered saline (PBS-0.145 M NaCl, 0.01 M phosphate buffer, pH 7.4) (31). The crude extract was fractionated with ammonium sulfate at 40% saturation. The precipitate obtained was redissolved in PBS and refractionated at 40% saturation with ammonium sulfate. The second precipitate was resuspended in PBS and observed to agglutinate strongly human erythrocytes, blood type O. Rabbits were immunized with this twice precipitated anti-H lectin in Freund's complete adjuvant. They were subsequently bled, and sera obtained were stored at −20°C. Antisera to human A and B substance were prepared from commercial anti-A and anti-B sera (Ortho Diagnostic Div., Raritan, N. J.). The antisera were precipitated with 50% ammonium sulfate at 4°C, redissolved at one-third original volume in PBS, pH 7.4, and dialyzed exhaustively against PBS at 4°C. The titer of these antisera was $>1:500$. Rabbit antihuman IgM serum was purchased commercially (Behring Diagnostics, Inc., Woodbury, N. Y.). Rabbit antihuman IgG serum was prepared as previously described (29). For examination by immunofluorescence technique, the IgG fraction of samples of appropriate antisera were conjugated with fluorescein isothiocyanate according to methods previously described (9, 29).

Preparation of tissues and cells for immunofluorescence studies. Segments of umbilical cords measuring approximately 0.5 cm in thickness were embedded in gelatin, frozen in a dry ice-acetone bath (32), and stored at −20°C. Sections were cut at 2.5 μm in a cryostat, mounted on glass slides, fixed in acetone for 10 min, and air dried. Cover slips containing cultured cells were fixed in acetone for 10 min and stored at −20°C.

Sections of umbilical cord or cover slips bearing endothelial cells were treated with antisera by direct and indirect techniques for immunofluorescence according to methods previously described (9).

Mixed cell agglutination reaction. The mixed cell agglutination reaction was performed by an adaption of the method of Coombs, Franks, Gurner, Polley, and Richards (33). Cells were grown as described above in plastic Petri dishes. Human anti-A and anti-B sera were prepared as described above. Anti-H lectin was prepared from *Ulex europaeus* seeds as described above and had a titer of 1:32–64 against adult type O red cells. Citrated blood of appropriate type was obtained, and the red cells were washed three times in PBS, resuspended to 2% packed cell volume in PBS, and stored at 4°C. The cells were used within 2 days of venipuncture. To test cultured cells, the medium was removed, and the Petri dishes were washed twice with 2 ml of diluent buffer-PBS containing 0.5% bovine serum albumin (Miles Laboratories, Inc., Kankakee, Ill.) and 0.011 M glucose. The Petri dishes were then incubated with 0.1% collagenase-0.01% EDTA in diluent buffer for 15 min at 37°C. The loosened cells were triturated to separate clumps and then washed twice with cold diluent buffer containing 0.02% EDTA by centrifugation at 200 *g* for 5 min. The resultant cell suspension was counted in a hemocytometer, and the suspension was adjusted to 10^5 cells/ml. 0.1 ml of cultured cell suspension was added to 0.1 ml of appropriate antisera, and the mixture was incubated at 4°C for 1 h. The cells were washed in diluent buffer six times (anti-A, anti-B sera) or four times (lectin) and resuspended in 0.4 ml of diluent buffer containing 0.02% EDTA. One drop of a 2% red cell suspension in PBS was added, and this mixture incubated at 20°C for 5 min. This mixture was centrifuged for 2 min at 200 *g*, most of the supernate was removed, the cells were resuspended gently in the remaining supernate and this suspension was smeared on microscope slides. The smears were air dried and stained with Wright-Giemsa, and then two groups of 500 cultured cells each were counted for rosette formation. A cultured cell was counted as a rosette only when it was surrounded by a collar of red cells, i.e., usually more than 10–15 red cells/tested cell.

Cell proliferation studies. Cultured endothelial cells were harvested with 0.01% EDTA-0.1% collagenase and washed twice with TC 199-20% FCS. The cells were plated out into 35-mm Petri dishes at an initial concentration of 100,000 cells/dish. At intervals the cells were harvested as above and counted in a hemocytometer.

RESULTS

Effects of collagenase digestion on umbilical vein histology. In samples of umbilical cord taken before collagenase treatment, endothelial cells could be seen lining the vein's interior (Fig. 1*a*). In specimens taken after collagenase treatment, the endothelial cell lining was selectively lost leaving the basement membrane and underlying structures intact (Fig. 1*b*). This finding was typical for veins that yielded only polygonal cells that were subsequently characterized as endothelial cells. If the collagenase incubation time was lengthened or the cord deliberately damaged by clamping, then the basement membrane was digested, and underlying structures were disrupted.

Light microscopic studies. After 5 days in culture, endothelial cells (derived from undamaged umbilical veins) grew in confluent monolayers without a definable whorling pattern. The cells were homogenous, closely

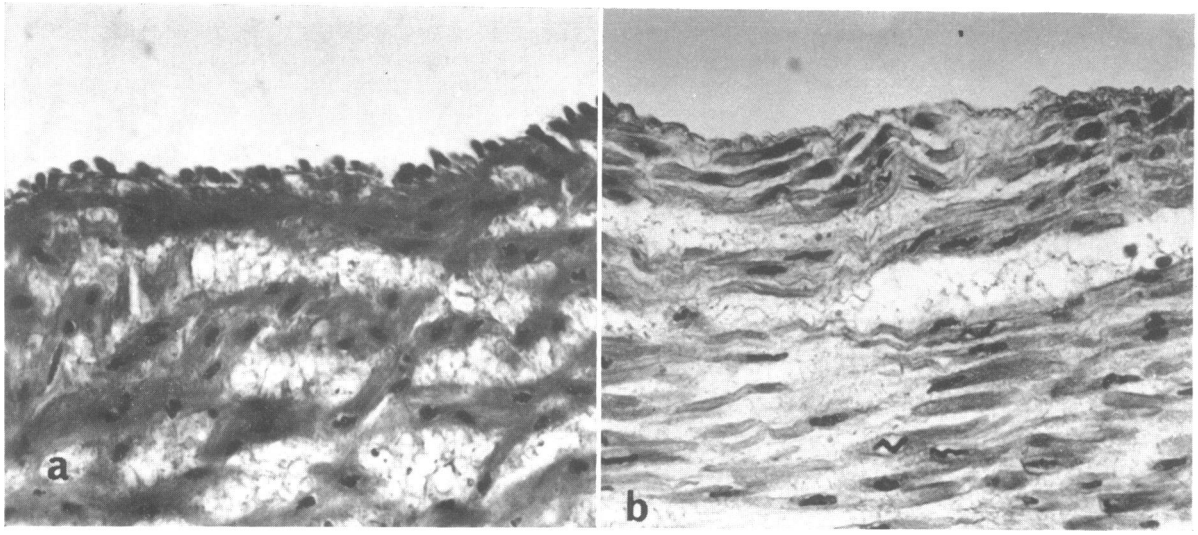


FIGURE 1 Photomicrograph of umbilical cord vein (*a*) before and (*b*) after collagenase treatment. Endothelial cells are present as a continuous layer in (*a*) and absent in (*b*). Hematoxylin and eosin (× 240).

opposed, large, and polygonal with an oval, centrally located nucleus and indistinct cell borders (Fig. 2). By phase contrast microscopy, cell borders were distinct and in some areas interdigitations appeared to connect separate cells. Cultured fibroblasts were microscopically quite unlike the cultured endothelial cells. Fibroblasts in confluent areas grew close to one another in parallel arrays with whorling and multiple overlapping layers. The cells were long, slender, and spindle-shaped with distinct cell borders. Phase contrast microscopy of cells, subsequently identified by electron microscopy (*vide infra*) as smooth muscle cells, revealed features very much like fibroblasts, i.e., long, slender cells that grew in parallel arrays with multiple overlapping layers. These smooth muscle cells, however, did not form whorls as did fibroblasts. These cells were morphologically similar to cultured smooth muscle cells as described by Ross (25).

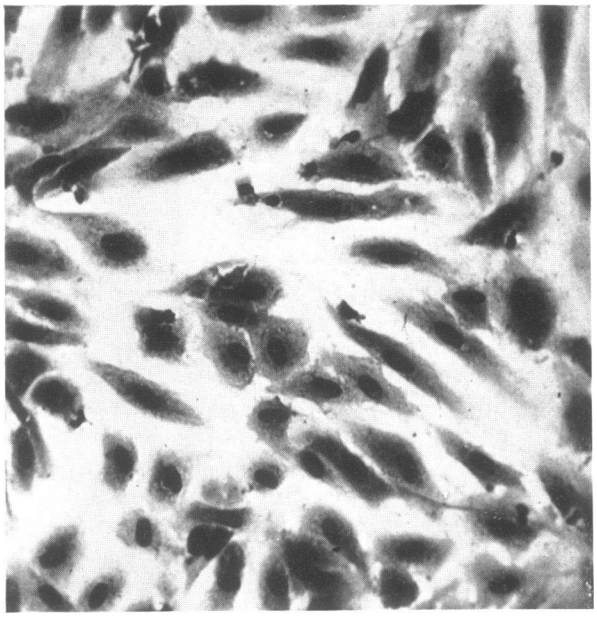


FIGURE 2 Photomicrograph of 5-day old cultured endothelial cells. These cells are a homogeneous population of large, closely opposed, polygonal cells with a centrally located nucleus and indistinct cell borders. Hematoxylin and eosin (× 190).

Observation of cultured endothelial cells for periods up to 5 mo revealed no evident transformation to spindle-shaped cells, i.e., fibroblasts or smooth muscle cells. When spindle-shaped cells appeared and overgrew the cultured endothelial cells, they originated from clumps of cells recognizably distinct from cultured endothelial cells even at the beginning of the culture. When examined by transmission electron microscopy, spindle cells appearing in these circumstances were identified as smooth muscle cells. This overgrowth by smooth muscle cells occurred only if the cord was deliberately damaged before collagenase treatment by repeatedly clamping it with a hemostat or if the collagenase incubation time was prolonged to 1 h. Histologic sections of the umbilical vein under these circumstances showed damaged subendothelium.

Scanning and transmission electron microscopy. When viewed in the scanning electron microscope, cultured endothelial cells were homogeneous. They were large (30 × 50 μm), flat cells that grew in a monolayer and

had distinct cell boundaries (Fig. 3*a*) and prominent nuclei. Many cells had numerous blebs and pits apparently on or near their surfaces; these were most prominent in the perinuclear region or near the edge of the cell. In contrast, cultured fibroblasts were spindle shaped and grew in parallel arrays. Nuclei of these cells were often not seen, and the previously described blebs and pits were not as numerous (Fig. 3*b*).

By transmission electron microscopy, endothelial cells seen *in situ* in umbilical cord veins were found to have the features of endothelial cells at other sites. They were thin and flattened with pinocytotic vesicles and many cytoplasmic vesicles. The cytoplasm also contained clusters of free ribosomes, smooth and rough endoplasmic reticulum, clumps of coarse filaments, fine filaments, and prominent microtubules. In addition, the cytoplasm of these cells contained numerous oval- or rod-shaped bodies like those described by Weibel and Palade (34). No Weibel-Palade bodies were seen in *in situ* smooth muscle cells or fibroblasts.

Cultured endothelial cells were morphologically similar when viewed by transmission electron microscopy. The cytoplasm of these cells contained large numbers of vesicles, prominent Golgi, large quantities of smooth and rough endoplasmic reticulum, clusters of free ribosomes, bundles of fine filaments with occasional dense bodies, microtubules, and long, irregular mitochondria (Fig. 3*c*, 4*a*, and 4*b*). In some cells, cisternae of endoplasmic reticulum were dilated and contained granular material. In occasional cells, the cytoplasm contained structures resembling discoid bodies described by Weibel and Palade. Moreover, many cells contained rod-shaped cytoplasmic inclusions (Fig. 4*c*) identical with those described in endothelial cells by Weibel and Palade (34) and seen in *in situ* endothelial cells (Fig. 4*d*). In any given section of suspended endothelial cells, approximately 30% contained Weibel-Palade bodies.

When examined by transmission electron microscopy, cultured smooth muscle cells were homogeneous. These cells contained relatively few cytoplasmic organelles, and large portions of their cytoplasm were occupied by bundles of fine filaments. In many areas, dense bodies or so-called "attachment bodies" were present within the bundles of myofilaments. Some cells contained fewer filaments. The cytoplasm of these latter cells often contained large quantities of rough-surfaced endoplasmic reticulum, prominent Golgi, numerous long, often branched mitochondria, and many membrane-bound dense bodies. No Weibel-Palade bodies were seen in cultured smooth muscle cells.

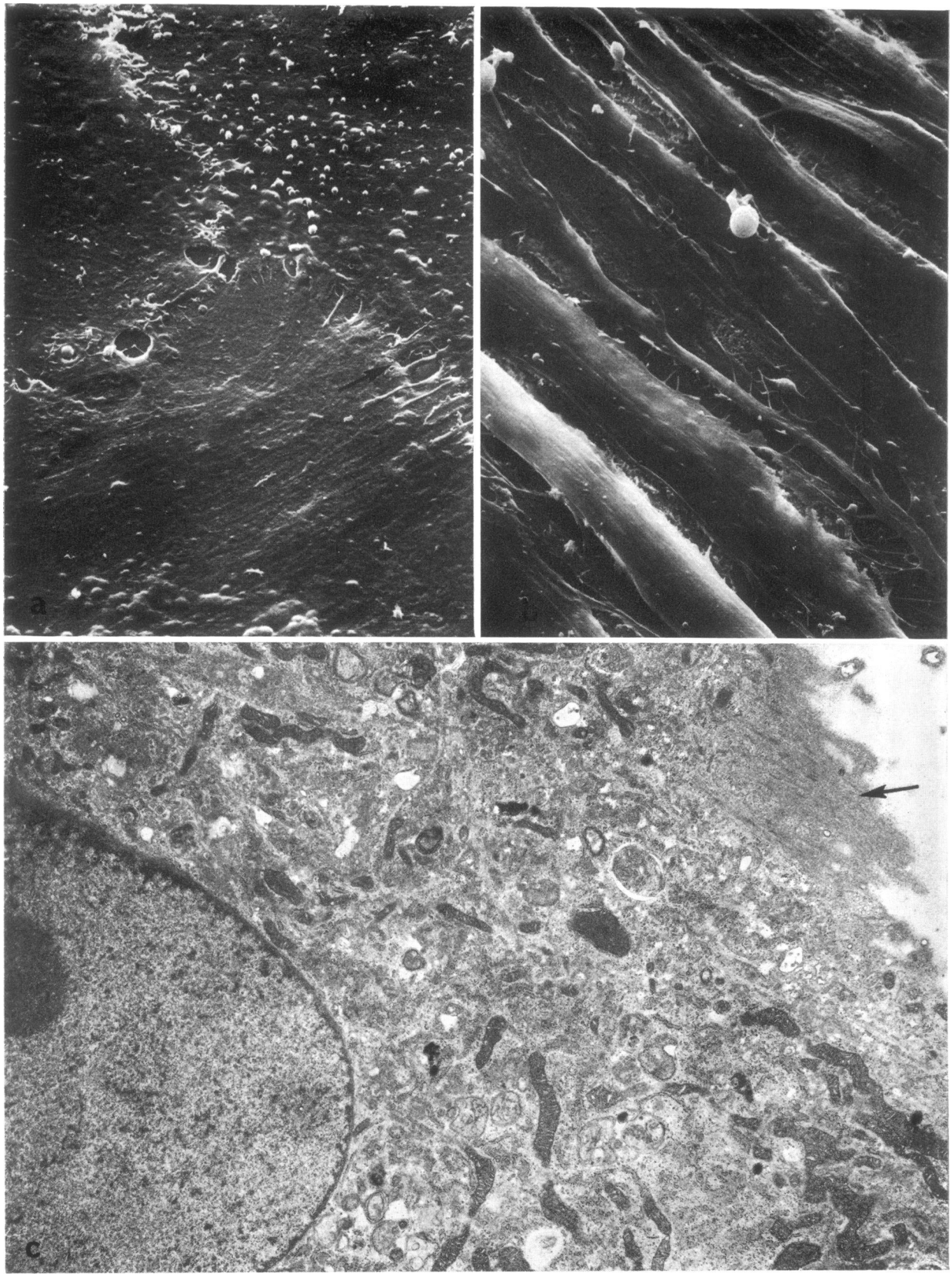
When examined by transmission electron microscopy, cultured fibroblasts were homogeneous. The cells contained rough-surfaced endoplasmic reticulum, variable amounts of fine myofilaments, pinocytotic vesicles, Golgi complexes, and clusters of free ribosomes. No Weibel-Palade bodies were seen in cultured fibroblasts.

Immunofluorescence studies demonstrating actomyosin in endothelial cells. When sections of umbilical cord were treated with fluorescein-conjugated rabbit antisera to uterine actomyosin (F-AUAM) or to platelet actomyosin (F-AT) and examined by immunofluorescence microscopy, endothelial cells and smooth muscle cells of umbilical arteries and veins and mesothelial cells forming a single layer at the outermost periphery of cord sections were brilliantly immunofluorescent. Fibroblasts in the matrix of the cord stained minimally; the matrix of the cord did not stain. Cultured endothelial cells were also stained brilliantly by F-AUAM and F-AT. The same results were obtained when either umbilical cord sections or cultured endothelial cells were treated with AUAM or AT and then with fluorescein-conjugated antisera to rabbit IgG (Fig. 5). Rabbit antisera to human skeletal muscle actomyosin or to cardiac muscle actomyosin did not stain endothelial cells, smooth muscle cells, or mesothelial cells by either direct or indirect immunofluorescence techniques. These observations indicate that endothelial, smooth muscle, and mesothelial cells of umbilical cords and cultured endothelial cells contain actomyosin of smooth muscle type. When tissue cultures of human fibroblasts were stained with F-AUAM or F-AT, it was observed that cells in some cultures did not stain, whereas cells in other cultures stained variably. In general, staining of cultured fibroblasts by these reagents was always much less intense than that of endothelial cells, either cultured or *in situ*.

Immunofluorescent staining by antisera to platelet actomyosin of *in situ* endothelial, smooth muscle, or mesothelial cells, or of cultured endothelial cells was completely inhibited in both direct and indirect immunofluorescence studies by previous absorption with platelet actomyosin or with uterine actomyosin. Staining of these structures by antisera to uterine actomyosin was completely inhibited by previous absorption with uterine actomyosin and partly inhibited by previous absorption with platelet actomyosin. This difference in results of absorption experiments is due to partial, but not complete immunological identity between uterine and platelet actomyosin (9). Immunofluorescent staining in direct or indirect immunofluorescence studies by AT or AUAM was not inhibited by absorption with cardiac actomyosin, skeletal actomyosin, pooled red blood cells, pooled white blood cells, lyophilized human plasma, lyophilized human fibrinogen, or lyophilized human dermal collagen.[2]

Studies demonstrating ABH antigens on endothelial cells. In tissue sections, human endothelial cells con-

[2] Kindly provided by Doctors Sidney Rothbard and Robert Watson.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tain ABH antigen(s) appropriate to the tissue donor's blood type whereas connective tissue and smooth muscle lack this antigen system (35–37). The following studies were undertaken to determine whether cultured endothelial cells contain ABH antigens.

Lectin, a plant agglutinin derived from *Ulex europaeus,* was used to demonstrate H antigens. When sections of umbilical cord were treated with anti-H lectin followed by rabbit antisera to anti-H lectin and finally by fluorescein-conjugated guinea pig or goat antisera to rabbit IgG, endothelial cells and mesothelial cells were brilliantly immunofluorescent indicating that they contain H antigen. Smooth muscle cells of blood vessels and fibroblasts in cord matrix stained minimally. Cord matrix did not stain. Approximately one-third of endothelial cells cultured on cover slips were observed to be brilliantly immunofluorescent when similarly treated, although many morphologically similar cells on the same cover slips were not stained at all, indicating that the cell population was heterogeneous with respect to content of blood group antigen. Immunofluorescent staining of endothelial and mesothelial cells was inhibited when anti-H lectin was absorbed with human type O erythrocytes before use. The minimal staining of smooth muscle cells and fibroblasts was not inhibited by absorption of anti-H lectin with type O erythrocytes suggesting the presence in low titer of contaminating antibody unrelated to the H system.

When sections of umbilical cord or endothelial cells cultured on cover slips were treated with anti-A or anti-B sera followed by a mixture of fluorescein-conjugated antihuman IgG and IgM, no immunofluorescent staining was seen.

A more sensitive technique, the mixed-cell agglutination reaction, was used to test cultured cells for A, B, and H antigens (Table I). Column A represents the percentage of rosettes formed when the test cells were reacted with anti-A sera, washed, and then reacted with A erythrocytes. As control, B and O erythrocytes were substituted for A erythrocytes; these controls were always negative. The same procedure with the obvious control substitutions was used for the B system. Because the anti-H lectin had a titer of 1:4–1:8 against A and B erythrocytes, control reactions with A or B erythrocytes were not performed; the A and B systems served as the control. Three separate lines of cultured fibroblasts contained no ABH antigens. Three separate lines of cultured smooth muscle cells contained no ABH antigens. In contrast, 12 different endothelial cell lines contained ABH antigens appropriate to the fetal donor's blood type. Endothelial cell H antigen was expressed strongly (high percentage of rosettes) whereas A and B antigens were generally weaker but present.

TABLE I

Mixed Cell Agglutination Reaction

Cell line–Blood type			Indicator system A	B	O
F*	(3)‡	A,O§	0‖	0	0
SM	(3)	A,A,O	0	0	0
EC	(2)	O	0	0	36.4
EC	(6)	A	5.7	0	22.6
EC	(2)	B	0	11.6	22.2
EC	(2)	AB	5.9	5.0	5.4

* F, fibroblasts; SM, smooth muscle cells; EC, endothelial cells.

‡ Number of separate experiments.

§ Blood type of donor of one fibroblast line was not determined.

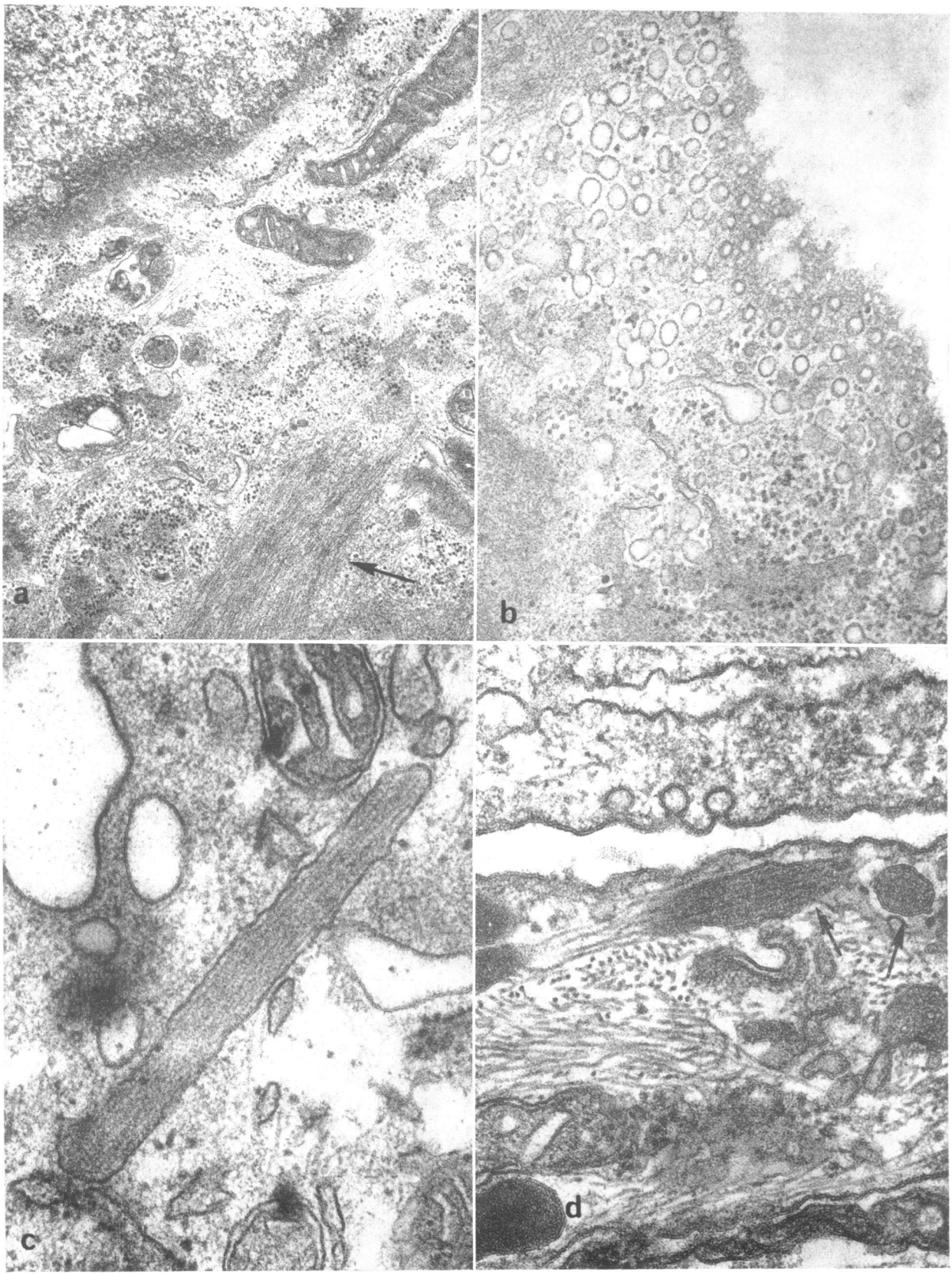
‖ Percentage rosette formation.

Cell proliferation. When primary endothelial cell cultures were harvested with 0.01% EDTA-0.1% collagenase and the cells diluted 1:4–1:8, it was possible to subculture cells for a period up to 5 mo and serially pass them up to 10 times.[3] Cell growth was rapid enough to allow subculture every 10–14 days. Few cells were lost during serial passage because there was approximately 75% attachment of the newly inoculated cells. Throughout the entire culture period the cells were fed twice a week with complete changes of fresh culture medium. Cell proliferation studies performed during the logarithmic phase of cell growth (Fig. 6) demonstrated a doubling time of 92 h.

After 3 mo in culture and five serial passages, there were no significant changes noted in the cultured cells. Light microscopic and transmission and scanning electron microscopic studies showed that: (*a*) the cellular morphology was unchanged, (*b*) the cells contained the same proportion of Weibel-Palade bodies, (*c*) the cells formed a homogeneous population with no transformation into other cell types. Immunologic studies

[3] At the time of the completion of the manuscript.

FIGURE 3 (*a*) Scanning electron photomicrograph of cultured endothelial cells, 7-days old. The cells grow in a monolayer and are large, flat, and polygonal. They are closely opposed and intercellular interdigitations are visible (arrow). (×2,620.) (*b*) Scanning electron photomicrograph of cultured skin fibroblasts. In contrast to endothelial cells, these cells are spindle shaped and grow in parallel arrays. (×2,150.) (*c*) Transmission electron photomicrograph of a cultured endothelial cell sectioned *en face.* Note numerous long, irregular mitochondria and fine filaments immediately beneath the cell membrane (arrow). (×8,450.)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showed the presence of smooth muscle actomyosin and ABH antigens in approximately the same degree as in primary cultures.

DISCUSSION

The goal of experiments presented here was to culture endothelial cells from human umbilical veins and to identify them unequivocally as endothelial cells according to morphologic, immunohistologic, and serologic criteria.

Endothelial cells cultured from umbilical veins grew as a homogeneous monolayer of large polygonal cells with ill-defined borders. Fibroblasts cultured from adult human skin and smooth muscle cells cultured from the media of umbilical veins grew as long, slender, spindle-shaped cells that formed parallel arrays and, often, overlapping layers. These cultural differences between endothelial cells on one hand and fibroblasts and smooth muscle cells on the other were entirely consistent from culture to culture.

Study of cultured cells by transmission electron microscopy revealed that endothelial cells (but not fibroblasts or smooth muscle cells) contained rod-shaped, cytoplasmic organelles like those first described in endothelial cells *in situ* by Weibel and Palade. Study of umbilical vein sections by transmission electron microscopy revealed that only endothelial cells contained Weibel-Palade bodies (34); they were not present in fibroblasts, smooth muscle cells, or other tissue components. It is reasonable to conclude from these observations that the cells isolated from umbilical veins and grown in culture were, indeed, endothelial cells. Endothelial cells cultured from umbilical veins as well as those *in situ* were also observed to contain filaments that resembled similar structures seen in smooth muscle cells (25). These filaments were present in the cytoplasm of umbilical vein smooth muscle cells and to a much lesser extent in the cytoplasm of some cultured fibroblasts.

In previous communications from our laboratories it has been demonstrated that human platelet actomyosin (thrombosthenin) and human uterine actomyosin are antigenically similar to each other but entirely distinct from actomyosin of striated skeletal or cardiac muscle (9). Antisera to either thrombosthenin (AT) or to uterine actomyosin (AUAM) will, by direct or indirect immunofluorescent technique, stain smooth muscle cells of uterus, blood vessels, and gut and endothelial cells of arteries, veins, liver sinusoids, and capillaries in heart and skeletal muscle (9, 29, 38). In experiments presented here, AT or AUAM intensely stained endothelial, smooth muscle, and mesothelial cells in sections of umbilical cords. Fibroblasts in the stroma of the umbilical cord were stained minimally to moderately. Cultured endothelial cells were also stained intensely by AT and AUAM. Some cultures of human fibroblasts contained cells that were stained by AT or AUAM while other cultures did not. These observations are in harmony with previous reports indicating that endothelial cells of certain vascular beds contain contractile protein of smooth muscle type. They are also in harmony with the observations of Majno, Shea, and Levanthal (39) and of Robertson and Khairallah (40) demonstrating contraction of endothelial cells in response to vasoactive amines or peptides.

The fact that some fibroblasts in culture were stained by AT or AUAM whereas others were not, parallels the results of experiments in which sections of various human tissues were treated with AT or AUAM (9). In general, fibroblasts were not stained by these reagents. However, fibroblasts in granulation tissue and fibroblasts in the stroma of the umbilical cord were stained. These and other observations (41, 42) indicate that under certain conditions, i.e. healing wounds, the fetal state, etc., fibroblasts have the capacity to synthesize contractile protein and assume contractile properties (9, 41). Obviously, staining of cultured endothelial cells by AT or AUAM does not identify these cells as endothelial cells since certain other cells also contain contractile protein of smooth muscle type. However, staining of cultured umbilical vein endothelial cells by AT or AUAM does demonstrate that the cultured cells possess a significant characteristic of *in situ* umbilical vein endothelial cells.

A number of investigators have demonstrated that human blood vessel endothelial cells (but not smooth muscle cells or fibroblasts) contain major blood group (ABH) antigens (35–37, 43–47). Recently, however, Szulman demonstrated that endothelial cells of human umbilical veins contained only H antigens but not A or B antigens, except in the few centimeters adjacent to the fetus where umbilical vein endothelium contained A or B in addition to H (48). In experiments presented here, H antigen (but not A or B antigen) was demonstrable in umbilical vein endothelial cells *in situ* and in vitro by immunofluorescence microscopy. H and a smaller amount of A and/or B antigens were demonstrable, however, in cultured endothelial cells by the mixed-cell agglutination reaction, a tech-

FIGURE 4 Transmission electron photomicrographs. (*a*) Cultured endothelial cell containing bundles of fine filaments (arrow) and many custers of free ribosomes. (× 31,000.) (*b*) Cultured endothelial cell containing large numbers of cytoplasmic vesicles and many free ribosomes. (× 54,600.) (*c*) Weibel-Palade body in cultured endothelial cell. (× 95,000.) (*d*) Umbilical vein endothelial cell *in situ*. Note endocytotic vesicles, coarse filaments, and longitudinal and cross-sections of Weibe-Palade bodies (arrows). (× 109,300.)

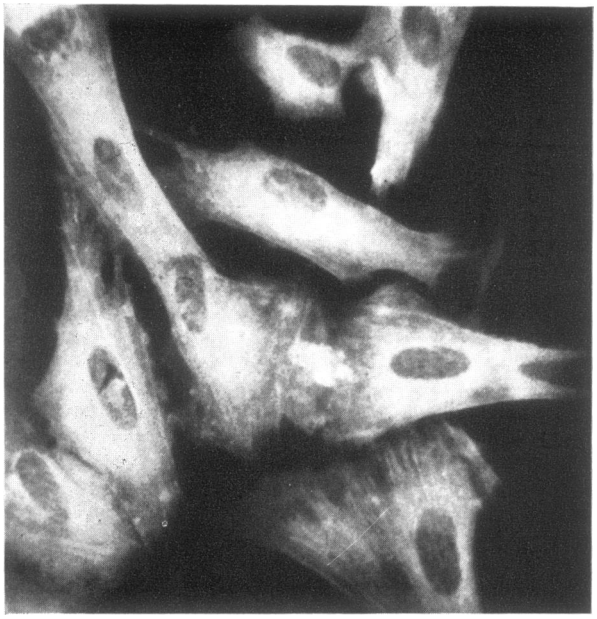

FIGURE 5 Immunofluorescent photomicrograph of cultured endothelial cells. Cells were incubated with rabbit antithrombosthenin (AT) followed by fluoresceinated guinea pig antirabbit gamma globulin. Positive immunofluorescence indicates the presence of smooth muscle actomyosin. (× 600.)

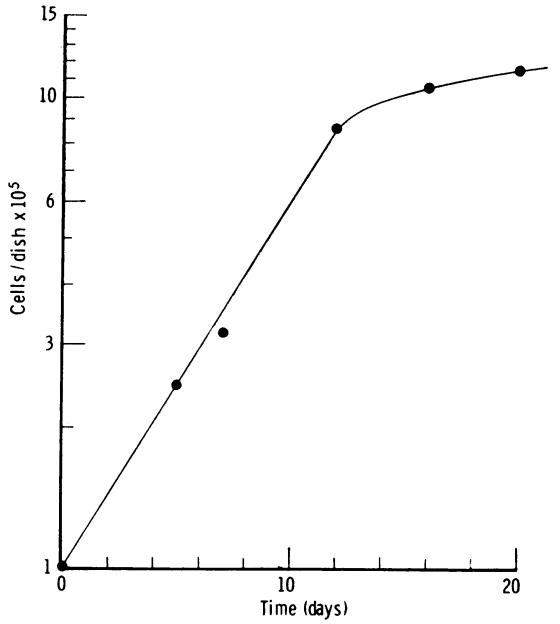

FIGURE 6 Growth curve of human endothelial cells in vitro. Each point represents the mean of three or four samples. SD was less than 10% of the mean for all points.

nique more sensitive than immunofluorescence (44, 47). Blood group antigens were not demonstrable in smooth muscle cells or in fibroblasts *in situ* or in vitro by either immunofluorescence microscopy or mixed-cell agglutination reactions. Therefore, demonstration of blood group antigen in cultured endothelium clearly differentiates these cells from either smooth muscle cells or fibroblasts.

The finding of a low percentage of cultured endothelial cells with detectable A and B antigen is consistent with Szulman's observation that A and B antigens are only found in the first few centimeters of umbilical vein adjacent to the fetus. Since the cord utilized in our studies measured 10–30 cm in length, it is obvious that only a limited percentage of cells would contain these antigens. The heterogeneous distribution of H antigen is also consistent with the observation that only 20–60% of cells in tissue culture contain H antigen when tested by the mixed-cell agglutination technique (43, 49–52). This heterogeneity is not due to a nonhomogeneous population because it also appears in clones of cells arising from a single cell (50).

Consideration of the data presented in these studies permits a clear differentiation of endothelial cells cultured from umbilical veins from other cultured cells on the basis of morphologic and immunologic criteria (Table II). The cell morphology, growth pattern, and the

TABLE II

Differentiating Characteristics of Endothelial Cells

Cell type	Cell morphology	Growth pattern	Weibel-Palade bodies	Myofilaments	Immunofluorescent staining with AT or AUAM	Presence of major blood group antigens
Endothelial cell	Polygonal	Single layer No pattern	+	+	+	+
Vascular smooth muscle cell	Spindle	Multi-layered Parallel arrays	−	+	+	−
Fibroblast	Spindle	Multi-layered Parallel arrays Whorled	−	+ or − variable	+ or − variable	−

presence of Weibel-Palade bodies, myofilaments, smooth muscle actomyosins, and ABH antigens delineates these cells from vascular smooth muscle and fibroblasts. In addition, as demonstrated in the accompanying paper (53), cultured endothelial cells contain antihemophilic factor antigen.

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